ANAEROBIC RECOVERY OF ASCARIS EGGS FROM X–IRRADIATION [1]

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Lea (1947) in his classic work on radiation biology has pointed out in his analysis of the work of Henshaw (1940) that the recovery of unfertilized, x-irradiated eggs of *Arbacia* is probably not due to diffusion out of the egg of inhibitory substances; the correlation between recovery rate and oxygen uptake suggests that the effect of the radiation is to destroy some nuclear constituent, and recovery consists in the re-formation of this constituent as a result of the metabolic activity of the cell. The rate of oxygen uptake is presumably an indication of the general level of metabolic activity, and in *Arbacia* eggs appears to vary in different stages of the egg in much the same way as does whatever reaction is responsible for recovery. It does not follow necessarily that the rates of recovery in different organisms will be proportional to their respective rates of oxygen uptake. Some organisms have, in fact, been shown to consume oxygen at appreciable rates after irradiation although they do not show any recovery.

The eggs of *Ascaris lumbricoides suum* possess certain advantages for a test of the question whether oxygen is necessary for recovery from x-irradiation. Since they are facultative anaerobes they can be held for long periods of time in anaerobic conditions. Even at optimal temperatures for normal development, under anaerobic conditions the eggs do not develop. If recovery should occur during the enforced anaerobic metabolism, not only would the necessity of oxygen uptake for recovery be disproved for *Ascaris* eggs, but some other possible mechanism of recovery would be suggested. The present paper complements a preliminary report (Pahl and Bachofer, 1954).

Materials and Methods

A stock of eggs of *Ascaris lumbricoides suum* in the one-cell stage was prepared according to methods already described by Bachofer and Pahl (1955). The source of x-rays was a beryllium-window tube operated at 100 kvp. and 8 ma., without added filtration. Each irradiated sample consisted of 10^5 eggs suspended in one ml. of distilled water and placed in an open, flat-bottom vial 2.7 cm. in diameter. The egg suspension was approximately 1.8 mm. deep with the eggs resting on the bottom during the exposure. The dose was calculated to be 12,000 r/min. at the center of the irradiated layer of eggs. This value was determined by exposing ferrous ammonium sulfate as a dosimeter to a 325-curie cobalt-60 source of gamma rays, as described by Weiss (1952). *Ascaris* eggs were then exposed to the gamma rays under the same conditions, and the biological response was correlated with dose.

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Aliquots of the same sample of eggs were then exposed to the x-ray beam, and from their response the output of the x-ray tube was determined. All subsequent exposures to x-rays were carried out under identical conditions at the same dose rate.

Each irradiated sample was diluted 20-fold with *Ascaris* physiological saline solution (Baldwin and Moyle, 1947) immediately after irradiation. Aliquots of two ml. each were de-oxygenated by bubbling specially purified nitrogen through the saline solution containing the eggs. The eggs were then placed at the appropriate incubation temperatures. After certain designated incubation periods, the seals were broken and the supernatant de-oxygenated fluid was drawn off while the eggs remained settled on the bottom. *Ascaris* saline, in equilibrium with air, was then added and incubation completed at 30° C. The same procedure was followed for non-irradiated controls.

TABLE I

Effect of post-irradiation anaerobic treatment at 15° C., 20° C., and 30° C. over a period of two weeks on the survival of x-irradiated Ascaris eggs. Dose: 28,000 r

Post-irradiation treatment		Per cent survival			
		Days under anaerobic conditions			
		0	1	7	14
30° C.	Aerated	47.5	45.0	47.3	48.8
	De-oxygenated	47.3	58.0	57.3	60.3
20° C.	Aerated	49.8	46.5	43.0	35.0
	De-oxygenated	45.0	48.3	50.0	38.8
15° C.	Aerated	48.8	42.0	36.0	27.8
	De-oxygenated	47.5	51.8	45.5	34.3

The two criteria used to determine the effects of irradiation and anaerobic treatment were the rate of first cleavage and the percentage of eggs which developed to the motile embryo stage. The term "survival" is used to designate the development of irradiated eggs into motile embryos.

RESULTS

Table I shows the results of irradiation of *Ascaris* eggs which were de-oxygenated immediately after irradiation and stored at temperatures of 15° C., 20° C., and 30° C. After periods of 1, 7, and 14 days at these temperatures, the de-oxygenated samples were aerated and incubated at 30° C. The results show that at any given temperature there was a higher survival for irradiated eggs given anaerobic treatment than for those incubated only aerobically. Keeping the eggs under anaerobic conditions for more than one day did not increase survival. Irradiated eggs kept at 30° C. throughout the entire post-irradiation period showed the highest survivals

TABLE II

Per cent survival of x-irradiated Ascaris eggs as affected by anaerobic treatment immediately after exposure

Dose in roentgens	Per cent survival				
	Days of post-irradiation anaerobic treatment				
	0	½	1	2	7
0	97.5	96.8	97.0	97.3	96.6
28,000	47.3	56.7	58.0	57.1	57.3
40,000	26.9	36.3	36.4	36.6	36.4
48,000	12.5	20.8	21.1	21.7	22.0
60,000	2.5	6.7	6.4	6.8	6.6

for both anaerobic and aerobic treatment. This confirms a previous study of the authors (Bachofer and Pahl, 1955) on post-irradiation temperature treatment of *Ascaris* eggs. In view of this, all other experiments were performed at 30° C., which is approximately the optimal incubation temperature.

Table II shows that anaerobiosis is able to bring about recovery over a wide dose range, even when untreated samples give survivals as low as 2.5%. When there was no anaerobiosis there was no recovery, recovery being represented by the difference in survival between the first column (where there was no anaerobiosis) and the other columns (where there was anaerobiosis). The critical period of recovery is shown to occur during the first 24 hours of anaerobiosis, since survivals for 12 hours of treatment are not increased appreciably for longer periods of treatment.

The effect of delaying the anaerobic treatment after irradiation was next investigated, with both cleavage delay and survival as criteria of recovery. The results summarized in Table III for cleavage delay show that the eggs must be de-oxygenated before 15 hours have elapsed after irradiation in order to procure recovery. The low values in the column for *no delay* in anaerobic treatment indicate the greatest recovery. Conversely, in Table IV, the high values in the column for *no delay* in anaerobic treatment indicate the greatest recovery. The crucial period of approximately 15 hours, therefore, affects both cleavage delay and survival.

TABLE III

50% cleavage time in hours for x-irradiated Ascaris eggs as affected by 24-hour anaerobic treatment initiated at various intervals after exposure

Dose in roentgens	No anaerobic treatment	Delay before anaerobic treatment		
		0 hrs.	15 hrs.	24 hrs.
	50% cleavage time in hours			
0	40.0	39.5	40.3	40.0
24,000	53.1	46.7	52.7	53.1
40,000	58.4	51.4	57.7	58.2
48,000	60.0	53.4	59.4	59.5

The recovery phenomena summarized above have been verified over a dose range of 24 to 48 kr, both for delay of cleavage and for survival.

Since anaerobic conditions during irradiation give high protection to *Ascaris* eggs, an experiment was designed to test whether post-irradiation anaerobic recovery could be secured with eggs which had been protected by anaerobic conditions during irradiation. When a dose of 60,000 r was delivered to one sample of eggs

TABLE IV

Per cent survival of x-irradiated Ascaris eggs as affected by a 24-hour anaerobic treatment begun at various intervals after exposure

Dose in roentgens	No anaerobic treatment	Delay of anaerobic treatment			
		0 hrs.	15 hrs.	24 hrs.	60 hrs.
	Per cent survival				
0	97.0	97.0	97.3	97.5	97.0
24,000	63.5	73.9	64.8	62.3	62.0
40,000	26.9	36.4	27.2	27.6	25.8
48,000	12.5	21.1	11.8	12.5	12.0

that was in equilibrium with air and to another similar sample that was under anaerobic conditions, the survival was increased in both cases by post-irradiation anaerobiosis. These increases in survival were duplicated when treatment for 1½ hours in 0.1 *M* KCN was substituted for post-irradiation anaerobiosis. Immediately after the period of exposure to cyanide, the eggs were washed by centrifugation and incubated in *Ascaris* saline. The results in Table V clearly indicate that the same pattern of protection can be obtained with cyanide as with anaerobiosis.

TABLE V

Effect of 24-hour post-irradiation treatments immediately following the irradiation of Ascaris eggs under different conditions during irradiation. Dose: 60,000 r

Treatment after irradiation	Treatment during irradiation	
	In equilibrium with air	Anaerobic
	Per cent survival	
Aerobic	2.5	74.3
Anaerobic	6.4	82.1
KCN	6.5	82.2

In other studies (unpublished results) the authors have established that cyanide inhibits the oxygen consumption of *Ascaris* eggs. Cyanide, however, is a general inhibitor of respiratory cycles whether they include the cytochrome system or not. To demonstrate that *Ascaris* eggs do have a cytochrome system, they were subjected to the light-reversal inhibition test of carbon monoxide by use of a Warburg respirometer adapted to this purpose. The eggs showed the same rate of oxygen consumption in air and in a 5% oxygen–95% nitrogen mixture. When CO replaced the nitrogen, however, there was an immediate and persistent drop in oxygen

consumption. Within a few minutes this leveled off at approximately 40% of normal consumption. In the presence of light this value rose to 75% of normal consumption.

Discussion

Numerous studies on the effect of anaerobiosis and other factors *during* irradiation are not comparable to the present investigation, since the present study utilizes anaerobiosis *after* irradiation and is therefore concerned with recovery processes. Although a number of post-irradiation treatments have delayed the expression of injury or decreased its rate of development, most of them have had no effect on the final outcome. In work with mice, Bacq *et al.* (1950) found that NaCN given immediately after irradiation only delayed mortality, and they concluded that cyanide was ineffective when given after irradiation. Bachofer (1956) has shown that post-irradiation anaerobiosis of x-irradiated *Ascaris* eggs restores in part the normal rate of pronuclear fusion, which is slowed down considerably by x-irradiation; the restoration is a genuine recovery and is attributable to the period of anaerobiosis.

The problem proposed by Lea (1947), as to whether the recovery of irradiated invertebrate eggs demands oxygen uptake or whether this uptake is a mere concomitant action, has been solved for *Ascaris* eggs. The facultative anaerobic nature of *Ascaris* eggs makes possible a complete elimination of free oxygen during incubation at optimum temperature. When x-irradiated eggs were subjected to post-irradiation anaerobiosis, their power of recovery surpassed that of x-irradiated aerated eggs as shown by decreased cleavage time and by higher survival (Tables I–V). It has been established, therefore, that oxygen is not necessary for recovery in this case.

A possible mechanism to be considered is whether this recovery could be attributed to the concomitant delay in cleavage brought about by anaerobic conditions. In studies concerned with cell cleavage, Schjeide and Allen (1951) found that tadpole hematopoietic cells appear to be susceptible to x-rays in direct proportion to the amount of cell division allowed to proceed following the irradiation period. Recovery of irradiated *Arbacia* eggs (Henshaw, 1940) was obtained only if they were kept unfertilized; as the time between irradiation and fertilization was shortened, the recovery was likewise decreased. In unirradiated *Arbacia* eggs cleavage begins at optimal temperatures within an hour after fertilization. Cytological observations (Bachofer, 1956) show that all eggs of *Ascaris lumbricoides* removed from the terminal 25 mm. of the uteri, in which the sperm has entered the egg, are in the pronuclear stage. Upon incubation at optimal temperature, pronuclear fusion begins slowly and precedes first cleavage by approximately 1½ hours. The eggs begin first cleavage only after 25 to 30 hours of incubation at optimal temperature, and achieve 50% cleavage after 40 hours. Since anaerobiosis had to be initiated before 15 hours had elapsed after irradiation in order to secure recovery, and anaerobic recovery was reduced if the treatment was delayed even a few hours following irradiation, it appears that the recovery process in question is not directly associated with the delay of first cleavage. Furthermore, if delaying the time of cleavage were the important factor in recovery, it would be expected that the survival of the irradiated eggs which were de-oxygenated and placed at the various sub-optimal temperatures would have remained at the same peak as those placed at 30° C. The progressive decrease in survival of both the aerated and anaerobically incubated eggs kept for

increasing lengths of time at temperatures lower than optimal (Table I) indicates that some factor other than cleavage delay is responsible.

Further evidence that cleavage delay is not the contributing condition for recovery is shown by the fact that the anaerobic incubation facilitates recovery only during the first 15 hours of this treatment (Table II). Likewise, eggs which have been allowed to incubate aerobically for 15 hours before being de-oxygenated give no evidence of recovery as indicated by cleavage delay (Table III) and survival (Table IV). During this 15 hours of aerobic incubation the eggs have not yet begun their first division. It appears that one must look to other conditions than delay of cleavage to explain the recovery.

It should be borne in mind that cellular activity, including cell cleavage, is necessary in most cases to demonstrate the injury, since the injury is latent. Postponement of cellular activity after irradiation may not involve recovery; once cellular activity is allowed to proceed the damage may be manifested. If the damage is as great as that which would have been manifested by permitting cellular activity to proceed immediately after irradiation, then there was no genuine recovery. True recovery was reported by Cook (1939) for survival of irradiated eggs of *Ascaris megalocephala* held at low temperatures after irradiation, but the opposite was found to be true for *Ascaris lumbricoides* (Bachofer and Pahl, 1955). Both studies agreed, however, in that post-irradiation treatment did not affect the time required for first cleavage. Pertinent to the present case, therefore, is the fact that forestalling cell cleavage and cellular activity after irradiation does not in itself produce genuine recovery.

The seat of the recovery from irradiation may involve various reactions of the respiratory cycle. The increased survival of irradiated eggs which have been subjected to cyanide or to anaerobiosis after irradiation suggests that the effects of irradiation operate to some extent through the cytochrome system, since both cyanide and anaerobiosis inhibit the cytochrome system. The mechanism of protection afforded by respiratory inhibitors may be either the prevention of the products of irradiation from reacting with the cytochromes or the prevention of the radiation-affected cytochromes from participating in the chain of reactions that normally bring about the observed effects of irradiation. Insofar as the cytochromes may be involved, the second possibility appears more pertinent in the present study, since the anaerobic condition would be too late, in time, to prevent a highly activated radiation product from reacting with the cytochromes, but it could prevent the affected cytochromes from reacting further.

There is, however, a function more important than holding the cytochromes in abeyance (Bachofer, 1956). It appears that checking aerobic metabolism permits anaerobic metabolism to restore essential molecules needed for normal development. The fact that recovery was greater for eggs held anaerobically at 30° C. than at sub-optimal temperatures indicates that anaerobic metabolism is associated with the recovery under consideration.

Summary

1. X-irradiation of *Ascaris lumbricoides suum* eggs produced delay of cell cleavage and reduced the percentage of eggs that completed embryogenesis. The time required for cleavage of irradiated eggs was reduced by an anaerobic treatment after

irradiation. The percentage of eggs that completed embryogenesis was increased by the same post-irradiation anaerobiosis. After the anaerobic treatment, eggs must be incubated aerobically since there is no perceptible development under anaerobiosis, although recovery takes place during this period. This recovery is greater at 30° C. than at sub-optimal temperatures.

2. Maximum recovery was obtained for eggs placed immediately after irradiation under anaerobiosis for periods of approximately 15 hours or more at 30° C. If the anaerobic treatment is delayed for 15 hours, the recovery is negligible.

3. Post-irradiation treatment with cyanide also fostered recovery from x-irradiation comparable to that secured with anaerobiosis.

4. Recovery was not due to delay of cleavage: the critical period for recovery took place long before cell division occurred even in air-saturated non-irradiated controls.

5. A cytochrome system in the eggs was demonstrated. The effects of cyanide treatment and anaerobiosis suggest that the mechanism of recovery may involve inhibition of the cytochrome system, which is prevented from participation in the reactions producing the expected deleterious effects of irradiation. There is, however, a positive contribution attributable to anaerobic metabolism, since recovery is greatest at optimal temperatures under anaerobiosis.

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